THE ESTIMATION OF HEPARIN AND SIMILAR SUBSTANCES IN HUMAN BLOOD AND TISSUES USING A COMBINED BIOLOGICAL AND COLORIMETRIC METHOD WITH PAPER ELECTROPHORETIC STUDIES

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Heparin and other acid mucopolysaccharides were extracted from various tissues by a modification of the method previously described (Bassiouni, 1953). The dye mucopolysaccharides complex obtained was resolved into its components with formamide: the acid mucopolysaccharides were precipitated with methyl alcohol in the presence of electrolyte (sodium chloride). A solution of the purified polysaccharide precipitate was bio-assayed for active heparin, and the remainder was either submitted to electrophoresis on paper or reprecipitated with the dye and measured colorimetrically.

Methods

Reagents.—The following are required:—

- (1) Sodium citrate, 3.8 g./100 ml.
- (2) Sodium chloride, 0.9 g./100 ml.
- (3) Sodium hydroxide, 0.2 N (Analar).
- (4) Ammonium sulphate (specific grade) dried, 6.5 g.
- (5) Dye solution, asymmetric dimethyl thionin or Azure A (Revector, Hopkin & Williams, Ltd.), 100 mg. dissolved in 100 ml. distilled water.
- (6) Hydrochloric acid, 0.01N.
- (7) Standard heparin, calcium salt of heparin, 99 units/mg. (Evans).
- (8) Buffered solvent, made up of 4 parts acetone, 2 parts distilled water, 2 parts of glycine-sodium hydroxide buffer consisting of 20 parts of molar glycine solution and 80 parts of normal sodium hydroxide solution.
- (9) Formamide, "pure" (L. Light & Co., Ltd., Colnbrook, Bucks).
- (10) Sodium chloride, saturated solution.
- (11) Methyl alcohol.
- (12) Calcium chloride solution, 0.2. Molar.
- (13) Phosphate buffer pH 7 made up of Na_2HPO_4 , 2H₂O M/15 305.5 ml. and 194.5 ml. KH_2PO_4 M/15.
- (14) Chlorobenzene.

Extraction.—Blood, 4 ml., is drawn, mixed with 1 ml. 3.8% sodium citrate, and centrifuged at 2,000 r.p.m. for 10 minutes: the plasma is then pipetted off, the cells washed with 2 ml. saline, and the wash added to the plasma. The plasma is made up to 7 ml. with distilled water in a conical pyrex flask of 25 ml. capacity; the white cell layer is pipetted off and suspended in another conical flask of the same capacity and containing 7 ml. distilled water. For tissue analysis, 1 g. of fresh tissue is weighed out, then cut into small pieces, macerated with powdered glass and transferred with 7 ml. distilled water into a pyrex conical flask of 25 ml. capacity.

To each 25-ml. flask, 0.5 ml. sodium hydroxide 2N is added; the flask is corked and warmed at 50° C. in a hot-water bath for 20 to 30 minutes with shaking from time to time; 6.5 g. ammonium sulphate is then added, the flask recorked, shaken vigorously, and left for five minutes. It is then warmed quickly in a boiling-water bath to 75° C., vigorously shaken and the contents transferred rapidly to fluted filter paper (Whatman 1, 7 cm. diameter). The filtrate is received into a thoroughly cleaned conical centrifuge tube of 12 ml. capacity, containing 1 ml. distilled water and 1 ml. dye solution. The tube is corked and inverted three times to mix, then left for an hour, being shaken from time to time; finally it is centrifuged for 45 minutes at 3,000 r.p.m. while capped. It is then decanted, the residue being washed first with 5 ml. 0.01N HCl and then with 2 ml. distilled water. Now the precipitate is resolved into its components by dissolving in 0.25 ml. formamide, helped by warming to 50° C. for a few minutes; it is then centrifuged and the supernatant liquid taken off with a micropipette and transferred to a clean tube. To it is added 0.25 ml. of saturated solution of sodium chloride and 2 ml. methyl alcohol, the mixture being centrifuged at 3,000 r.p.m. for 20 minutes.

The precipitate is re-extracted with 0.25 ml. saturated solution of sodium chloride; to the extract 0.25 ml. distilled water and 2 ml. methyl alcohol

are added and the mixture centrifuged for 20 minutes. The tube is decanted for five minutes, the mouth wiped dry and the tube dried *in vacuo*. The precipitate is dissolved in 0.5 ml. distilled water and 0.1 ml. of the solution used for bio-assay to which 1 ml. plasma is added.

FIG. 1.—Inhibitory effect of heparin on coagulation of human plasma.

Biological Estimation.—The biological estimation is based on an observation that within a certain range of concentration of heparin added to re-calcified sheep plasma, the logarithm of the clotting time is proportional to the amount of heparin added (Fig. 1). Fresh sheep blood collected from the slaughter-house, 400 ml., is received in a glass flask containing 100 ml. 3.8% sodium citrate and the plasma is separated and kept in 20-ml.-capacity bottles in a refrigerator. The amount of calcium chloride M/5 solution necessary for optimum recalcification of 1 ml. plasma is determined.

The heparin clotting inhibition curve is obtained by preparing a series of conical tubes (thoroughly cleaned, 12 ml. capacity) each containing 1 ml. of sheep plasma.

To one tube is added 0.1 ml. distilled water to serve as a blank; to the rest is added 0.1 ml. solution of standard heparin in distilled water containing amounts varying from 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 inter-

national units. The tubes are corked and kept in a water bath at 25° C. for 10 minutes, then 0.2 ml. of M/5 calcium chloride solution is added to each tube and clotting observed every half minute. The method depends on recording the clotting time at a certain fixed stage in all the tubes. This is taken to be such that when the tube is tilted to an angle of 45° the surface of the plasma does not tilt at all, keeping strictly at right angles to the tube. The logarithm of clotting time is obtained and plotted against the amount of heparin added.

Human plasma from stored blood whose date of use has expired (30 days) can be used for the same purpose. It is noteworthy that the older the samples, whether sheep or human, the more sensitive they are to the action of heparin, most probably due to the fact that prothrombin concentration declines with storage, especially with rise of temperature, leading to less thrombin on recalcification. Thus the anti-thrombin action of heparin becomes more apparent. Fresh human plasma was found insensitive to such low concentrations of heparin.

Estimation of Unknown Sample.—The heparin content of the unknown sample is read off on the heparin inhibition curve. When the unknown gives a higher clotting time than the standard tubes, 0.05 ml. of unknown solution is made up to 0.1 ml. with distilled water and assayed again. Confirmatory experiments to assess the efficacy of this method of assay were done by comparing part of an extract, whose biological activity had been determined, with a known standard of the same strength. Both gave exactly the same clotting time. For each experiment a standard curve should be done.

Colorimetric Estimation.—A standard curve is prepared by mixing 1 ml. of a diluted standard solution of heparin containing from 0.01 mg. up to 0.1 mg. with 1 ml. dye solution. This is then centrifuged for 30 minutes and the precipitate is washed successively with 5 ml. 0.01 N HCl and 2 ml. distilled water, then dissolved in 8 ml. of buffered solvent and is estimated electro-colorimetrically in a single cell spectrophotometer using a blue-green filter No. 623.

Estimation of Unknown Sample.—Of a dilution of the extract, 1 ml. is mixed with 1 ml. dye solution and the same procedure followed as for the standard curve.

Paper Electrophoresis of the Extract.—Electrophoresis of heparin and chondroitin sulphate on filter paper was carried out, using the apparatus and following the general procedure described by Consden and Stanier (1952). Whatman No. 1 filter paper was used with phosphate buffer pH 7 as the electrolyte and chlorobenzene as the organic solvent. The unknown heparin extract, the standards of heparin, and the chondroitin sulphate were each dissolved in 0.02 ml. distilled water, and applied to the filter paper as a thin band on the same level 0.5 cm. from the end near the cathode. The voltage was 300 v and the potential gradient approximately 7.3 v/cm. applied

for two hours, after which the paper was removed and dried at 100° C. It was then sprayed with an alcoholic solution of Azure A 0.033% w/v in methyl alcohol. (Chondroitin sulphate was kindly supplied by Dr. E. J. Holborrow prepared from human cartilage by the method of Einbinder and Schubert, 1950.)

Staining with an alcoholic solution of the dye gave bands with clear, well-defined margins, whereas staining with an aqueous dye solution gave bands with ragged and ill-defined margins (Consden, 1953).

Recoveries

The recoveries and losses at various stages of the procedure were studied in detail by extracting a known amount of heparin from a blank reagent. The results (Tables I, II, Fig. 2) show that heating,

TABLE I
RECOVERIES OF 70 µG. HEPARIN (7 UNITS) AT TWO STAGES OF PROCEDURE

Procedure	Recovery at Stage of Obtaining Heparin-dye Complex (µg.)	Recovery (Biological Method) after Removal of Dye and Precipitation of Heparin (units)
Extraction from plasma	50-47	2.5
In blank undergoing whole procedure	52	3.1
In blank with no NaOH added	50	3.0
In blank with no heating and no filtration	60	3.3

TABLE II
PERCENTAGE OF ADDED HEPARIN RECOVERED AT THE TWO STAGES OF PROCEDURE

Added Heparin		Recovery at Stage of Obtaining Heparin-dye Complex		Recovery after Removal of Dye and Precipitation of Heparin with Methyl Alcohol	
µg.	Units	µg.	%	Units	%
1.0	0.1	0.7	70	0	0
2.0	0.2	2.0	100	0	0
3.0	0.3	3.0	100	0	0
4.0	0.4	4.0	100	0.0-0.05	0-12
5.0	0.5	5.0	100	0.17	32
6.0	0.6	5.4	90	0.21	35
10.0	1.0	9.0	90	0.34	34
30.0	3.0	22.0	70	1.0	33
50.0	5.0	35.0	70	1.6	33
60.0	6.0	45.0	80	2.2	37
70.0	7.0	50.0	80	2.5	36
100.0	10.0	80.0	80	5.0	50

filtration, and washing the heparin-dye complex cause a total loss of about 15% whether 0.5 ml. of 2N NaOH were added or not ; with extraction of the same amount of heparin from the 4 ml. of stored plasma the loss rises to 20%, i.e., 5% more than in the blank reagent. About half of the remaining 80% (40% of the total) is lost during the

TABLE III
VARIOUS VALUES FOR STANDARD HEPARIN AND CHONDROITIN SULPHATE

	Anti-coagulant Units	Colorimetric Value (mg.)	A/C Value*	Paper Electrophoresis	
				Mobility	Colour of Stained Bands
1 mg. heparin	100	1.0	100	100	Alpha form
1 mg. chondroitin sulphate	0.2	0.5	0.4	87.2	Beta form

* A/C value = $\frac{\text{anticoagulant activity in units}}{\text{colorimetric value in mg.}}$

FIG. 2.—Recoveries: colorimetric × and biological •.

formamide separation of heparin from the dye. This leaves us with 40 to 50% of the starting material ; the proportion of this loss is so constant within the range 1-20 units that it may be allowed for in the calculation of the result without serious error. The figures given in Tables V and VI are corrected ones, the directly obtained values being multiplied by 2 for both the anticoagulant and the colorimetric estimation in order to allow for the inevitable loss. This loss in the last step is a material one as reprecipitation with the dye proves. It seems inevitable with very low concentration of heparin, and efforts to achieve higher recovery were not successful. The method cannot detect an initial concentration of heparin less than 0.5 units/2 ml. (5 µg./2 ml. plasma) since, by the stage of freeing the heparin from the dye, the 0.5 unit becomes 0.17 unit (1.7 µg.) which is the minimum amount detectable biologically.

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TABLE IV
VARIOUS VALUES FOR ADULT AND NEWLY BORN
HUMAN TISSUES

Tissue	Anti-coagulant (Units/1 g. Tissue)	Colori- metric Value (mg./1g. Tissue)	A/C Value	Paper Electrophoresis	
				Mobi- lity	Colour of Wet Stained Band
*Adult human:					
lung	11.0	0.118	92.0	94.7	Gamma- beta forms
heart	4.2	0.070	60.0		
liver	3.9	0.070	55.0		
spleen	3.3	0.057	58.0		
kidney	10.25	0.118	87.0		
brain	2.4	0.040	60.0		
†Connective tissue..	5.3	0.360	15.0	84-87.2	Beta forms
Cartilage	2.75	0.600	4.6		
Aorta	1.8	0.280	6.3		
‡Severely atheroma- tous aorta	1.6	0.190	8.3		
Blood, 450 ml.:					
R.B.C.s	0	0	0		
W.B.C.s	0.8	0.080	10.0		
Plasma	0	0	0		

* From a woman aged 60: sudden death (ruptured heart).

† From fibrous capsule of knee-joint.

‡ From a case of diabetes and acromegaly.

|| From a one-week-old blood from the transfusion centre.

FIG. 3.—The values of the A/C ratio for various mixtures of heparin and chondroitin sulphate.

Results

The estimation of heparin-like substances in various adult and newly born tissues are shown in Tables IV and V. The highest content in the adult

TABLE V
RESULTS OF ESTIMATIONS IN TISSUES OF THE
NEWBORN*

Tissues	Anti-coagulant (Units)	Colori- metric Value (mg.)	A/C Value	Paper Electrophoresis	
				Mobility	Colour of Wet Stained Band
Lung ..	2.5	0.100	25.0	90	Beta form
Heart ..	2.5	0.120	20.8		
Liver ..	2.1	0.100	21.0		
Spleen ..	2.6	0.080	32.5		
Kidney ..	2.5	0.080	31.2		

* From a case of complete spina bifida.

was found in the kidneys and lungs, being about three times the content in other tissues. In the newly born the concentration is low and shows nearly similar values in all the organs analysed.

The plasma from 4 ml. blood samples taken from healthy adults (10 men and four women, aged 20-66) gave negative results biologically. The extraction of the larger amounts of blood (500 ml.) also gave negative results for both plasma and red blood cells; the white blood cells, however, gave an extract with very low anticoagulant activity and high colorimetric value.

The ratio of anticoagulant activity in units to the colorimetric value in mg. (A/C ratio) was found to be 100 for standard heparin and 0.4 for chondroitin sulphate (Table III). Mixtures of heparin and chondroitin sulphate in various proportions were estimated biologically and colorimetrically. The A/C ratio obtained for various mixtures was shown to be additive (Fig. 3). This ratio is very useful in indicating whether the obtained extracts contain mainly heparin-like or chondroitin sulphate-like substances. The A/C ratio in extracts from the aorta, white blood cells, and cartilage ranges between 6 and 10, similar to that of chondroitin sulphate, while extracts from human adult kidney, lung, heart, liver, spleen, and brain gave a ratio between 60 and 90, suggesting that they contain mainly heparin-like substances. In tissues from human newly born infants the A/C ratio was from 25 to 30, which would either mean a higher percentage of chondroitin sulphate or a heparin with a low anticoagulant activity.

On paper electrophoresis the band of chondroitin sulphate had a mobility of 87.2 relative to that of standard heparin as 100. The "heparin" extracted from

FIG. 4.—Electrophoresis of heparin from adult human kidney and spleen.

a.=standard heparin, 30 μ g. b.=heparin from kidney.
c.=heparin from spleen. d.=chondroitin sulphate, 120 μ g.

FIG. 5.—Electrophoresis of heparin from a newly born human kidney and heart.

a.=standard heparin. b.=heparin from heart.
c.=heparin from kidney. d.=chondroitin sulphate, 120 μ g.

human adult kidney, lung, heart, liver, spleen, and brain occupied an intermediate position between standard heparin and chondroitin sulphate (Fig. 4). The "heparin" from tissues of the newly born infant occupied a position much nearer to the chondroitin sulphate band (Fig. 5) while the adult aorta and white blood cell extracts gave bands on the same level as chondroitin sulphate (Fig. 6).

The metachromasia of the stained bands on filter paper while wet showed also distinct differences; the band of standard heparin was red, that of human adult heparin was reddish violet, while that of the newly born heparin was violet, as well as that of chondroitin sulphate.

The metachromasia was ascribed by Lison (1936) to the sulphate content of the mucopolysaccharide, and later thought by Michaelis and

FIG. 6.—Electrophoresis of aortic extracts on filter-paper.

a.=standard heparin, 25 μ g. b.=extract from normal aorta.
c.=extract from an atherosclerotic aorta. d.=chondroitin sulphuric acid, 25 μ g.

Granick (1945) and Michaelis (1947) to be due to the degree of polymerization of the dye when combined with various acid mucopolysaccharides, being red in trimers γ -form, violet in dimers β -form, and blue in monomers α -form.

These differences therefore suggest the possibility that the heparin-like substances of the tissues in the newly born are less esterified or less polymerized than in the adult. A higher percentage of chondroitin sulphate, as suggested by the A/C ratio, cannot explain the mobility differences. The electrophoretic results suggest that extracts of cartilage, aorta, and white blood cells contain a substance, or substances, with properties similar to chondroitin sulphate.

Summary

A micro-method is described for the extraction of heparin and chondroitin sulphate from 1 g. of various human tissues and their estimation biologically and colorimetrically. The ratio of anticoagulant activity in units to the colorimetric value in mg. (A/C ratio) can indicate whether the extract

is heparin or chondroitin sulphate or a mixture of both.

Extracts were also run on paper electrophoresis giving additional information from the rate of mobility and the colour of stained bands.

The results of estimations of extracts from adult and newly born tissues showed distinct differences between them in their heparin content, A/C ratio, and electrophoretic mobility.

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